

## Real-time PCR for the early detection and quantification of *Coxiella burnetii* as an alternative to the murine bioassay

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### ABSTRACT

Real-time PCR was used to analyze archived blood from non-human primates (NHP) and fluid samples originating from a well-controlled Q fever vaccine efficacy trial. The PCR targets were the IS1111 element and the *com1* gene of *Coxiella burnetii*. Data from that previous study were used to evaluate real-time PCR as an alternative to the use of sero-conversion by mouse bioassay for both quantification and early detection of *C. burnetii* bacteria. Real-time PCR and the mouse bioassay exhibited no statistical difference in quantifying the number of microorganisms delivered in the aerosol challenge dose. The presence of *C. burnetii* in peripheral blood of non-human primates was detected by real-time PCR as early after exposure as the mouse bioassay with results available within hours instead of weeks. This study demonstrates that real-time PCR has the ability to replace the mouse bioassay to measure dosage and monitor infection of *C. burnetii* in a non-human primate model.

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### 1. Introduction

*Coxiella burnetii*, the etiologic agent for the zoonotic disease Q fever, is a fastidious obligate intracellular bacterium found world-wide in sheep, goats, and cattle [1]. Agricultural workers who handle fluid and tissues of infected animals are at the greatest risk of direct exposure. Indirect exposure results from a highly infective spore-like form that persists in soil for months and is easily transmissible by airborne dissemination. With either route of exposure disease can result from as little as one cell [2–4]. Q fever acquired from inhalation of contaminated dust or aerosols is a major public health concern in areas of the world where *C. burnetii* is endemic [5]. Recent reports indicate Q fever in humans may not be recognized by clinicians and is actually on the increase in the United States [6], with more than 30 cases reported among US soldiers returning from the Middle East [7]. *C. burnetii* is also listed as a Category B priority pathogen and classified as Risk Group 3 by the Centers for Disease Control and Prevention. Although rarely fatal, acute Q fever is a debilitating flu-like illness with highly

variable and non-specific symptoms lasting from 1 to 6 weeks. A recently released report stresses the potential of an attack using a bioweapon on the citizens of the United States of America [8]. Airborne dispersal of even a relatively low dose of *C. burnetii* could sicken thousands, contaminate food or water supplies and cause panic in a heavily populated area. The disease disguised as a benign head-cold with flu-like symptoms would quickly inundate medical healthcare facilities [9].

Culturing the agent requires a living host and is both time consuming and hazardous. Antibiotic treatment can significantly diminish or even prevent illness when administered within a narrow window ranging from late in the incubation period to the third day of illness, but is much less effective after that time [10,11]. Most antibody diagnostic techniques are retrospective due to the time-frame for sero-conversion (3–4 weeks post infection), rendering them useless for timely treatment. In addition, the existing vaccines for Q fever have not been approved by the U.S. Food and Drug Administration for human use. Previous reports of detection of *C. burnetii* DNA by real-time PCR were validated based on cell culture isolates [12] or on clinical samples from naturally occurring infections of unknown dose and exposure date [13,14]. The two-fold purpose of the current study was to use real-time PCR to quantify the aerosol exposure dosage received by non-human primates (NHPs) during a Q fever vaccine efficacy trial and to

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14. ABSTRACT <b>Real-time PCR was used to analyze archived blood and fluid samples originating from a well-controlled Q fever vaccine efficacy trial. The PCR targets were the IS1111 element and the com1 gene of Coxiella burnetii. Data from that previous study were used to evaluate real-time PCR as an alternative to the use of sero-conversion by mouse bio-assay for both early detection and quantification of C. burnetii bacteria. The presence of C. burnetii in peripheral blood of non-human primates was detected by real-time PCR as early after exposure as the mouse bioassay with results available within hours instead of weeks. In a separate experiment, real-time PCR and the mouse bioassay exhibited no statistical difference in the number of microorganisms delivered in the aerosol challenge dose. Research animals are both costly and subject to stringent regulations so the judicious use of archived samples conserved biological as well as financial resources. This study demonstrates that real-time PCR has the ability to replace the mouse bioassay to measure dosage and monitor infection of C. burnetii in a non-human primate model.</b>					
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**Table 1**  
Primer and probe sequences of *Coxiella burnetii*-specific real-time PCR assays.

Target	Primer/ Probe	Sequences (5' → 3')	Amplicon (bp)	Final Conc (μM)	MgCl <sub>2</sub> (mM)
IS1111	IS1111-F801	AAT TTC ATC GTT CCC GGC AG	101	0.5	5
	IS1111-R901	GCC GCG TTT ACT AAT CCC CA		0.5	
	IS1111-p822S-MGB	6FAM - TGT CGG CGT TTA TTG		0.1	
		G - MGBNFQ			
com1-a	Cbcom1-F738	CAG CCG CTA AAC AAG GAA	91	0.5	5
		AAT ATT			
	Cbcom1-R828	GCG GTT TGA AGG GTG ATT TG		0.5	
	Cbcom1-p763-MGB	6FAM - TGC TTT CCA CGA CGC		0.2	
		G - MGBNFQ			
com1-b	Cbcom1-F822	AAA CCG CAG AAA AAG TAG	81	0.5	5
		GAT TAA AT			
	Cbcom1-R902	CAC GCA GTT GTT TTT GGA		0.5	
		TAG C			
	Cbcom1-p862-MGB	6FAM - AAA AGA CAT GGA TAA		0.1	
		TCC - MGBNFQ			

determine when in the course of infection *C. burnetii* DNA can be detected in the blood of those NHPs. TaqMan-MGB based real-time PCR assays targeting the single copy *com1* outer membrane protein gene and the multiple copy IS1111 transposase element of *C. burnetii* were used to test archived vaccine study samples and compared with the mouse bioassay findings obtained at the time of the original trial.

## 2. Materials and methods

### 2.1. Study animals

#### 2.1.1. Non-human primates

The Q fever vaccine efficacy trial, completed in 1996 and reported in 2002 [15] exposed 50 cynomolgus monkeys (*Macaca fascicularis*) to an inhaled dose of approximately 10<sup>5</sup> virulent phase I Henzerling strain *C. burnetii* bacteria [16,17]. Ten of the 50 NHPs were non-vaccinated control animals and the source of data and samples used in this study. Anesthetized monkeys were challenged using a head-only monkey aerosol exposure apparatus in a class II safety cabinet in a BSL-3 containment facility with continuous sampling during the procedure by all glass impingers (AGIs) containing sterile saline [18–20]. Daily observations for symptoms of Q fever were recorded and peripheral blood samples were collected on alternate days from day zero (preexposure) through day 22 (post-challenge) with a final bleed on day 42.

#### 2.1.2. Mice

Six-week old female CD-1 mice (Harlan Sprague-Dawley Indianapolis, IN), were used to measure *C. burnetii* in AGI fluid dose [21] and to detect bacteremia in NHP blood samples.

### 2.2. Real-time PCR

The real-time PCR assay primers and TaqMan®-MGB probe sequences and master mix concentrations are listed in Table 1. Primers were synthesized by Invitrogen (Carlsbad, CA) and the probes were synthesized by PE Biosystems (Foster City, CA). Real-time PCR assays for the IS1111 element and *com1* gene were developed and optimized following the USAMRIID common chemistry protocol [22]. All real-time PCR assays were carried out in 20 μL volumes (15 μL master mix and 5 μL sample) and run on a Roche LightCycler (LC) instrument (Indianapolis, IN). Cycling conditions consisted of an initial step to denature the DNA at 96 °C for 2 min followed by 40 amplification cycles of 96 °C for 1 s and 60 °C for 20 s with fluorescence measured at 530 nm after each 20 s step.

#### 2.2.1. IS1111 and com1 targets

The TaqMan-MGB assay for the multiple copy IS1111 insertion element of *C. burnetii* identified by Hoover et al. [23] has previously been published [22]. The gene sequences for the highly conserved single copy outer membrane-associated *com1* protein of *C. burnetii* (GenBank Accession #s: AB004693; AB004694; AB004695; AB004696; AB004697; AB004698; AB004699; AB004700; AB004701; AB004702; AB004703; AB004704; AB004705; AB004706; AB004707; AB004708; AB004709; AB004710; AB004711; AB004712 [24]; AF317646; AF317647; AF318145; AF318146; AF318147; AF318148; AF318149; M88613 [25]; and Z11828) were selected as potential *C. burnetii*-specific single copy targets. All sequence alignments were performed using the EMBL-EBI ClustalW (1.82) Multiple Sequence Alignment Tool. Regions of 100% homology were used as target sequences for each potential assay.

#### 2.2.2. Development of standard curve for quantification of *C. burnetii*

A standard curve for the IS1111 assay was generated using an initial *C. burnetii* (strain Nine Mile), DNA concentration of 7.4 μg/μL measured using a Beckman DU640 spectrophotometer (Fullerton, CA). A ten-fold serial dilution, from 10 pg to 10 fg, was run in triplicate on the Roche LC 1.2 instrument to generate the standard curve. Purified nucleic acid of each sample was run on the LC 1.2 using the IS1111 assay with a *C. burnetii* DNA sample of known concentration included as a single point standard. Experimental data were imported into the Roche LC software version 4.0 for analysis. An external standard curve for IS1111 was applied and the concentration of each sample was calculated automatically by the *Absolute Quantification* module within the software. The theoretical number of genome equivalents (GE) was calculated from the length of the published sequence of the *C. burnetii* Nine Mile genome (1,995,275 bp) [26]. This calculation relied on the weight of G + C (42.6% for *C. burnetii*) to be equal to 618.14 Daltons and A + T to be 617.43 Daltons with a total genome weight of 1.232,546,134 × 10<sup>9</sup>

**Table 2**  
Placebo control NHP blood sample results by mouse bioassay and real-time PCR over the time-course of the aerosol challenge study.

Days post infection	0	2	4	6	8	10	12	14	16 <sup>c</sup>	18	20	22	42
Clinical Signs of Illness <sup>a</sup>	0/10	0/10	0/10	10/10	10/10	10/10	9/9 <sup>b</sup>	9/9	9/9	0/9	0/9	0/9	0/9
Mouse Bioassay	0/10	0/10	3/10	9/10	10/10	8/10	5/9	4/9	1/4	0/9	0/9	1/9	0/9
Real-time PCR IS1111	0/10	0/10	4/10	10/10	10/10	10/10	9/9	8/9	3/4	3/9	3/9	1/9	0/9
Real-time PCR com1-a	0/10	0/10	0/10	5/10	8/10	9/10	9/9	6/9	2/4	0/9	0/9	0/9	0/9
Real-time PCR com1-b	0/10	0/10	0/10	5/10	8/10	9/10	8/9	7/9	2/4	1/9	0/9	0/9	0/9

<sup>a</sup> Presence of at least 3 of the following: fever, radiological, respiratory, and/or behavioral changes, abnormal blood chemistries.

<sup>b</sup> One NHP succumbed to infection on day 10 post infection.

<sup>c</sup> Due to low hematocrit only 4 NHPs bled this date.

**Table 3**

Exclusivity panel: DNA from the following tested negative for all three real-time PCR assays.

<i>Acinetobacter baumannii</i>	<i>Brucella canis</i> (4 strains)	<i>Enterococcus faecalis</i> (2 strains)	<i>Pseudomonas putida</i>
<i>Acinetobacter lwoffii</i>	<i>Brucella maris</i> (2 strains)	<i>Escherichia coli</i> (3 strains)	<i>Ralstonia pickettii</i>
<i>Actinobacillus pleuropneumoniae</i>	<i>Brucella melitensis</i> (14 strains)	<i>Feline DNA</i>	<i>Rhizobium radiobacter</i>
<i>Actinomyces naeslundii</i>	<i>Brucella neotame</i> (3 strains)	<i>Francisella philomiragia</i>	<i>Saccharomyces cerevisiae</i>
<i>Alcaligenes faecalis</i>	<i>Brucella ovis</i> (2 strains)	<i>Francisella tularensis</i> (11 strains)	<i>Salmonella choleraesuis</i>
<i>Alcaligenes xylosoxydans</i>	<i>Brucella suis</i> (2 strains)	<i>Haemophilus actinomycetemcomitans</i>	<i>Salmonella enterica</i>
<i>Bacillus anthracis</i> (12 strains)	<i>Budvicia aquatica</i>	<i>Haemophilus influenzae</i> (2 strains)	<i>Serratia marcescens</i>
<i>Bacillus cereus</i> (4 strains)	<i>Burkholderia cepacia</i>	<i>Halobacterium</i> sp.	<i>Serratia odorifera</i>
<i>Bacillus coagulans</i>	<i>Burkholderia mallei</i>	<i>Human DNA</i>	<i>Shewanella oneidensis</i>
<i>Bacillus halodurans</i>	<i>Burkholderia pseudomallei</i>	<i>Klebsiella oxytoca</i>	<i>Shigella flexneri</i>
<i>Bacillus licheniformis</i>	<i>Campylobacter jejuni</i>	<i>Klebsiella pneumoniae</i> (2 strains)	<i>Shigella sonnei</i>
<i>Bacillus macerans</i>	<i>Candida albicans</i>	<i>Lactobacillus acidophilus</i>	<i>Staphylococcus aureus</i> (6 strains)
<i>Bacillus megaterium</i>	<i>Canine DNA</i>	<i>Lactobacillus jensenii</i>	<i>Staphylococcus epidermidis</i>
<i>Bacillus mycoides</i> (2 strains)	<i>Chryseobacterium meningosepticum</i>	<i>Legionella pneumophila</i>	<i>Staphylococcus hominis</i>
<i>Bacillus polymyxa</i>	<i>Citrobacter freundii</i>	<i>Listeria monocytogenes</i>	<i>Staphylococcus lugdunensis</i>
<i>Bacillus popilliae</i>	<i>Clostridium acetobutylicum</i>	<i>Mannheimia haemolytica</i>	<i>Staphylococcus saprophyticus</i>
<i>Bacillus</i> sp.	<i>Clostridium bifermentans</i>	<i>Moraxella catarrhalis</i>	<i>Staphylococcus schleiferi</i>
<i>Bacillus sphaericus</i>	<i>Clostridium botulinum</i> type A	<i>Moraxella lacunata</i>	<i>Staphylococcus xylosum</i>
<i>Bacillus staerothermophilus</i>	<i>Clostridium botulinum</i> type B (2 strains)	<i>Morganella morganii</i>	<i>Stenotrophomonas maltophilia</i>
<i>Bacillus subtilis</i> (4 strains)	<i>Clostridium botulinum</i> type B	<i>Murine DNA</i>	<i>Streptococcus anginosus</i>
<i>Bacillus thuringiensis</i> (4 strains)	<i>Clostridium botulinum</i> type F	<i>Mycobacterium gordonae</i>	<i>Streptococcus pneumoniae</i>
<i>Bacteroides distasonis</i>	<i>Clostridium difficile</i>	<i>Mycobacterium species</i>	<i>Streptococcus pyogenes</i> (2 strains)
<i>Bacteroides forsythus</i>	<i>Clostridium perfringens</i> (2 strains)	<i>Mycoplasma pneumoniae</i>	<i>Streptococcus species Group B</i>
<i>Bacteroides fragilis</i>	<i>Clostridium sordelli</i>	<i>Neisseria lactamica</i>	<i>Streptococcus species Group F2</i>
<i>Bartonella henselae</i>	<i>Clostridium sporogenes</i>	<i>Neisseria meningitidis</i>	<i>Ureaplasma urealyticum</i>
<i>Bifidobacterium infantis</i>	<i>Comamonas terrigena</i>	<i>Pantoea ananatis</i>	<i>Vaccinia virus</i>
<i>Bordetella bronchiseptica</i>	<i>Comamonas acidovorans</i>	<i>Pasteurella multocida</i>	<i>Vibrio cholerae</i>
<i>Bordetella parapertussis</i>	<i>Corynebacterium diphtheriae</i>	<i>Porcine DNA</i>	<i>Vibrio parahaemolyticus</i>
<i>Bordetella pertussis</i>	<i>Deinococcus radiodurans</i>	<i>Porphyromonas gingivalis</i>	<i>Yersinia enterocolitica</i>
<i>Borrelia burgdorferi</i>	<i>Enterobacter aerogenes</i> (2 strains)	<i>Propionibacterium acnes</i>	<i>Yersinia frederiksenii</i>
<i>Bovine DNA</i>	<i>Enterobacter agglomerans</i>	<i>Proteus mirabilis</i>	<i>Yersinia kristensenii</i> (2 strains)
<i>Brevibacterium brevis</i>	<i>Enterobacter cloacae</i>	<i>Proteus vulgaris</i>	<i>Yersinia pestis</i> (8 strains)
<i>Brevibacterium linens</i>	<i>Enterobacter dissolvens</i>	<i>Providencia stuartii</i>	<i>Yersinia pseudotuberculosis</i> (8 strains)
<i>Brucella abortus</i> (4 strains)	<i>Enterococcus durans</i>	<i>Pseudomonas aeruginosa</i> (2 strains)	<i>Yersinia ruckeri</i>

Daltons or approximately 2.047 fg. Sample curves were analyzed using “Second Derivative Maximum” with baseline adjustment set to Arithmetic.

### 2.3. Fluid and blood samples

#### 2.3.1. AGI fluid samples

Enumeration of bacteria in the aerosol challenge fluid sampled from the AGIs was originally performed using the mouse bioassay [21,27]. Serial dilutions of each AGI sample ( $10^{-1}$  through  $10^{-5}$ ) were prepared and 0.1 ml of each dilution was injected intraperitoneal (i.p.) into groups of five mice. Twenty-one days after injection the presence of phase I and II (IgG) antibodies to *C. burnetii* were determined by ELISA [21]. Calculations made using Most Probable Number from serial dilutions were normalized to reflect the number of organisms per ml of AGI fluid (Bacteriological Analytical Manual Online <http://www.cfsan.fda.gov/~ebam/bam-a2.html>). The remaining AGI samples were archived at  $-70^{\circ}\text{C}$ . DNA for real-time PCR was subsequently extracted from the 25 archived AGI samples using IsoCode Stix according to the manufacturer's recommendations (Schleicher & Schuell) with minor modifications [28].

#### 2.3.2. NHP blood samples

On each bleed day during the original study 0.1 ml of undiluted peripheral blood from individual NHPs was introduced into a single mouse by i.p. injection. Twenty-one days after injection the presence of phase I and II (IgG) antibodies to *C. burnetii* were determined by ELISA as described above. The remaining NHP blood samples were archived at  $-70^{\circ}\text{C}$ . For the current study, DNA for PCR reactions was extracted from selected archived NHP blood samples by the Qiagen QIAamp DNA Mini Kit method according to

the manufacturer's instructions (Qiagen Inc., Valencia, CA) with minor modifications [28].

## 3. Results

### 3.1. Detection of *C. burnetii* by real-time PCR

#### 3.1.1. Inclusivity and exclusivity

The real-time PCR assays for IS1111 and *com1* (*com1*-a and *com1*-b) demonstrated inclusivity for all available *C. burnetii* isolates including; Ohio, Nine Mile 7, M44, Henzerling, and California 1949. Exclusivity testing showed no cross-reactivity of any of the three real-time *C. burnetii* assays to a panel of DNAs (Table 3) that included organisms closely related to *C. burnetii*, various pathogenic bacteria known to produce clinical symptoms similar to those of Q fever, and potential environmental contaminants.

#### 3.1.2. Limits of detection

The IS1111 TaqMan-MGB assay reproducibly detected 10 fg (4.9 genome equivalents) of *C. burnetii* genomic DNA while each TaqMan-MGB *com1* assay reproducibly detected 50–75 fg (24.4–36.6 genome equivalents).

### 3.2. Quantitative PCR for *C. burnetii*

#### 3.2.1. Standard curve

The IS1111 standard curve generated using the Nine Mile strain, which contains 20 copies of the IS1111 element, had an error of 0.229, a slope of  $-3.545$ , an efficacy of 1.946, an efficiency of 95% and an  $R^2$  value of 0.9991 (data not shown). The standard curve analysis indicated that one picogram of *C. burnetii* DNA with a crossing threshold ( $C_t$ ) of 25 by second derivative analyses

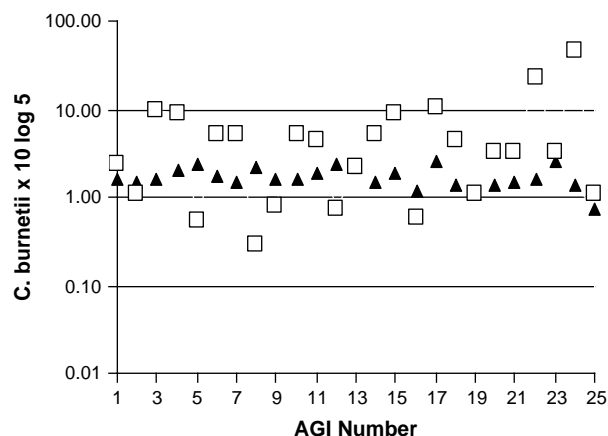


Fig. 1. Quantification of AGI fluid ▲ real-time IS1111 PCR assay □ mouse bio-assay.

represented approximately  $4.9 \times 10^5$  organisms. Results from the genomic DNA limit of detection experiments correlated linearly with a dynamic range of six orders of magnitude representing approximately 5–5,000,000 copies (data not shown).

### 3.2.2. AGI sample quantification

The number of *C. burnetii* organisms in the AGI samples calculated using mouse bioassay and real-time PCR are shown in Fig. 1. Dixon's Gap Test was used to identify outliers in AGIs 22 and 24 from the mouse bioassay results. These were removed from both sets for continuity making the total  $n = 23$  for analysis. In order to meet assumptions of normality, values were log 10-transformed prior to analysis. Results of Levene's test showed that variances were not homogenous between the mouse bioassay results and the PCR results ( $p = 0.0002$ ). A one-way analysis of variance (ANOVA) with Welch's correction for heterogeneous variances was used to analyze the data. Results of the ANOVA showed that the mouse bioassay results were not significantly different from the real-time PCR results,  $F(1,25) = 3.16$ ,  $p = 0.0823$ . The geometric mean for the mouse bioassay was determined to be  $2.5 \times 10^5$  with a standard error of the mean of 1.25. The real-time PCR geometric mean was  $1.7 \times 10^5$  with a standard error of the mean of 1.06. The mouse bioassay results showed approximately 4 times as much variation around the mean as the real-time PCR results.

### 3.3. Detection of *C. burnetii* in NHP blood samples

Results obtained with the three real-time PCR assays using DNA extracted from the archived peripheral blood samples of the 10 naïve NHPs in the original vaccine study are shown in Table 2. These are compared with mouse bioassay results and the clinical signs of disease recorded at each time point during that study. One of the NHPs died on day 10 post-challenge and five were not bled on day 16 due to low hematocrit ( $\leq 25$ ).

Positive results were obtained for over 96% of the real-time IS1111 PCR assays and 75% of real-time *com1* assays compared to less than 72% with the mouse bioassay for all of the blood samples drawn during the 10 day period of clinical illness ( $n = 52$ ).

For all individual NHP blood samples demonstrating positive results with the mouse bioassay ( $n = 41$ ), over 95% were positive for *C. burnetii* DNA with the PCR-based IS1111 assays ( $n = 39$ ). In every case these PCR-based IS1111 assays became positive either on the same day post exposure as the mouse bioassay or earlier. For all peripheral blood samples ( $n = 40$ ) collected from these NHPs during the optimal window for treatment (day 4 through day 10 post exposure), 85% tested positive with the real-time IS1111 PCR

assay, compared to 75% for the mouse bioassay and 55% for the real-time *com1* assays. During this time-frame, every naïve NHP in the study had at least 3 peripheral blood samples test positive for *C. burnetii* DNA by real-time IS1111 PCR assay.

## 4. Discussion and conclusions

In the present study, we describe the successful application of real-time PCR to the quantification of aerosol dose in AGI fluid and the early detection of *C. burnetii* in blood samples during the acute course of Q fever disease. This technique correlated well with the mouse bioassay results originally obtained for these samples during the Q fever vaccine study. Other techniques such as cell culture have proven to be inadequate for demonstrating low level bacteremia [29], whereas sero-conversion in mice has been shown to result from exposure to a single viable *C. burnetii* organism [27,30]. Serological methods, however, require weeks and research animals are both costly and subject to stringent regulations. The results we have reported in this paper originally required 748 mice and the complete vaccine efficacy trial required over of 1950 mice. DNA extracted from the archived NHP blood and AGI fluid samples from the Q fever vaccine study provided an extraordinarily valuable data set for evaluating currently available methods and equipment. This allowed us to demonstrate that real-time PCR has sensitivity equivalent to the mouse bioassay with results available in a clinically relevant time-frame.

The real-time PCR and the mouse bioassay exhibited no statistical difference in quantifying the number of microorganisms delivered in the aerosol challenge dose. The real-time PCR geometric mean for the AGI samples fell entirely within one standard deviation of the geometric mean of the mouse bioassay. The real-time PCR quantitative results were thus consistently within an acceptable range of the acknowledged true value of the starting concentration of *C. burnetii* obtained by the mouse bioassay, but with approximately four times less variation around the mean.

For detection of *C. burnetii* DNA in NHP blood the real-time IS1111 PCR assay proved to be more consistent than the mouse bioassay during the entire clinical course of the disease and during the critical window for treatment. Neither of the real-time *com1* assays detected bacteremia as early as the real-time IS1111 or the mouse bioassay, but performed as well as the bioassay after day 8 post infection. There was no significant difference between the two versions of the *com1* assay (*com1*-a and *com1*-b). The difference in sensitivity between the real-time assays probably reflects the multiple copies of IS1111 elements contrasted with the single copy *com1* gene.

The close correlation of the quantification of the AGI fluids by real-time PCR with the values obtained by an entirely different method 14 years ago reflects the soundness of the real-time technology, the robust nature of the targets and the remarkable persistence of *C. burnetii*. Even with some loss in recovery by the extraction of nucleic acids, the real-time PCR assay for IS1111 can be considered as an alternative to the mouse bioassay for both quantification in AGI fluid and detection in blood.

Real-time PCR assays targeting the *C. burnetii* IS1111 have been reported by several authors [5,12,14,31]. Recently, quantitative PCR using calibration curves developed from the cloned template of a segment of IS1111 from the Nine Mile strain of *C. burnetii* consistently detected 20 copies of the target sequence in close correlation with published sequence data [32]. There is little doubt that IS1111 element and the *com1* gene are appropriate targets for *C. burnetii*. However, in all previously referenced studies, details of exposure including time, route, infectious dose and time from exposure to illness were unknown. No preexposure or preclinical samples were available, making it impossible to evaluate when in

the course of the infection the organism could be detected by real-time PCR. In the current study the archived blood and fluid samples from the vaccine efficacy trial provided a unique sample set for which route and time of exposure, dose, and subsequent clinical course were all known. This allowed us to directly test the detection efficiency of real-time PCR assays during the entire clinical spectrum of acute Q fever and to demonstrate conclusively their utility for detection of *C. burnetii* in peripheral blood soon enough after infection to permit effective treatment.

Acute Q fever is a debilitating illness, but it is usually self-limiting, with only a small number of cases (<2%) progressing to chronic Q fever, a potentially devastating illness. Unfortunately because of the non-specific symptoms presented by Q fever, the acute disease is often under-diagnosed and underestimated as a public health risk in the U.S. [6], so that chronic disease resulting from *C. burnetii* infection may be the first time Q fever is diagnosed in some patients. Although endocarditis is the most common manifestation, a bewildering array of presentations has been reported, including hepatitis, pulmonary disease, osteomyelitis, and chronic wound infection with multisystem inflammatory dysfunction [33]. Fetal death with spontaneous abortion has also been described [5,34]. Timely detection of *C. burnetii* during the acute phase has the potential to reduce the impact of natural outbreaks or acts of bioterrorism, and protect the health of combat soldiers in high risk areas, and to prevent complications of a bizarre chronic illness with a baffling constellation of life-threatening symptoms. Real-time PCR assays represent a significant improvement over the cumbersome bioassay, providing equal or better results in hours rather than weeks while avoiding the use of laboratory animals.

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